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The Active-site Environment of Rhodopsin*

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The 11-*cis*-retinal binding site of rhodopsin is of great interest because it is buried in the membrane but yet must provide an environment for charged amino acids. In addition, the active-site lysine residue must be able to engage in rapid Schiff base formation with 11-*cis*-retinal at neutral and lower pH values. This requires that this lysine be unprotonated. We have begun to study the environment of the active-site lysine using a reporter group adducted to it. Non-active-site permethylated opsin was reacted with 5-nitrosalicylaldehyde, and the resulting Schiff base was permanently fixed by borohydride reduction. The stoichiometry of incorporation was one. This chromophoric and pH-sensitive reporter group affords information on the active-site environment of rhodopsin by determining the ionization constants of its ionizable groups at different pH values. The pH titration of the modified protein showed a single $pK_a \approx 7.8 \pm 0.19$ ascribable to the ionization of the phenol. The ionization of the modified lysine residue was not observed at all pH values studied. These studies are interpreted to mean that a negatively charged amino acid is propinquous to the active-site lysine residue and that this latter residue does not have an unusually low pK_a.

Rhodopsin is an integral membrane-bound protein containing an 11-*cis*-retinal chromophore covalently linked to an active-site lysine residue via Schiff base formation (1). Photolysis of rhodopsin leads to the *cis* to *trans* isomerization of the chromophore and the bleaching of the pigment to afford all-*trans*-retinal and opsin (2). One of the rhodopsin intermediates (R*) which occurs prior to bleaching, spectroscopically defined as metarhodopsin 2, catalyzes the exchange of GTP for GDP in a retinal G-protein called transducin (3, 4). This exchange initiates the visual transduction cascade, eventually leading to the closing of sodium channels in the plasma membrane of the rod outer segments and the hyperpolarization of the rod (5–7).

The active-site lysine of rhodopsin plays a significant role in the functioning of the pigment. This lysine residue must first be capable of engaging in Schiff base formation with 11-*cis*-retinal at neutral or even acidic pH values, thus forming rhodopsin. The essential lysine residue must be in the neutral unprotonated form prior to its reaction with 11-*cis*-retinal. The Schiff base that forms is thought to be partially or completely protonated (8). That this Schiff base is protonated is quite crucial in the formation of the early photochemical intermediates (9). In a related way, the protonated Schiff base is thought to be critical in photochemical energy storage by rhodopsin and in wavelength regulation (10, 11). Photochemical energy storage refers to the mechanism by which the photic energy absorbed by rhodopsin is largely stored as the potential energy of the protein, rather than being released as heat (12). The separation of the positively charged Schiff base from its counterion in the hydrophobic active-site region is thought to play an important role in the etiology of this effect (12). Wavelength regulation is concerned with the mechanism by which the various opsin molecules can interact with the same chromophore, 11-*cis*-retinal, producing pigments which absorb at different λ_{max} values (11). Electrostatic interactions between the protonated Schiff base and protein-bound counterions are thought to be at the basis of wavelength modulation. Color vision, as well, importantly depends on these interactions.

Not only must the Schiff base be protonated in rhodopsin and in the early photochemical intermediates, but the Schiff base also must lose its proton in order to form R*. This can be unequivocally demonstrated by observing the photochemical and biochemical behavior of active-site monomethylated rhodopsin (13). This latter rhodopsin derivative contains a permanently charged Schiff base (14). Photolysis of active-site methylated rhodopsin leads to the formation of the early photochemical intermediates up to and including metarhodopsin 1 (14). However, metarhodopsin 2, as measured spectroscopically, cannot form nor can R*, as biochemically defined (14).

Not only must the Schiff base of rhodopsin become deprotonated, but its lysine counterpart in opsin must also be deprotonated if Schiff base formation is to occur with 11-*cis*-retinal. Does this mean that the pK_a of the protonated lysine is unusually low? The pK_a of a typical ϵ-amino group of lysine is approximately 10.9 (15). At pH = 6.5, where opsin readily reacts with 11-*cis*-retinal, only 1 lysine in 10,000 would be deprotonated and able to form a Schiff base if the active-site lysine were typical. In this article, the question of the pK_a of the protonated form of the active-site lysine is addressed. Information on limits to the value of this pK_a will also provide information on the environment of the active site of opsin.

To approach this measurement, we made use of the fact that non-active-site permethylated opsin readily reacts with aromatic aldehydes in a pseudo-irreversible fashion, forming Schiff bases at the active-site lysine (Scheme 1 (16)). Although the previously studied *o*-salicylaldehyde (16) is not useful in reporting spectroscopic information, 5-nitrosalicylaldehyde is (17). In their classical studies on acetoacetate decarboxylase, Westheimer and colleagues measured the pK_a of the active lysine using the spectroscopic reporter group *p*-nitrophenol (17, 18). This reporter group can be adducted to a lysine

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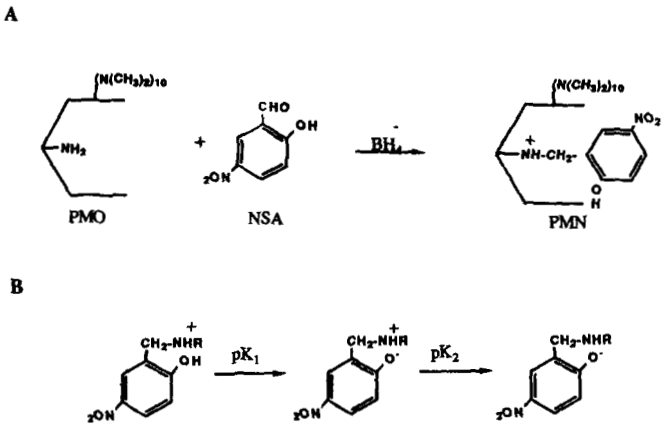
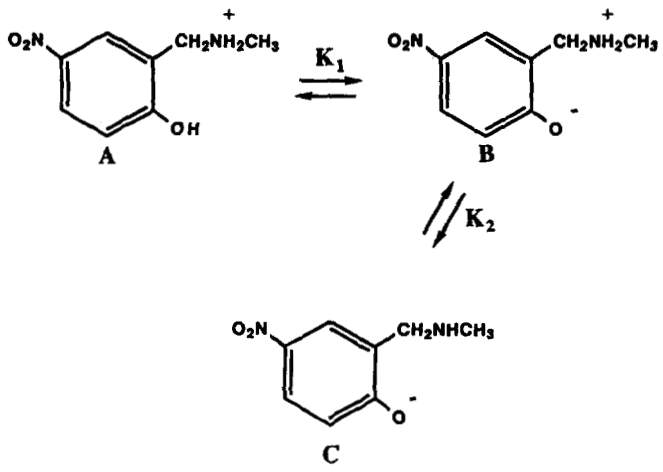


SCHEME 1. **Introduction of NSA reporter group into rhodopsin.**



SCHEME 2. **Ionization of the reporter group.**

residue by reacting the latter with 5-nitrosalicylaldehyde followed by borohydride reduction (Scheme 1 (18)). The nitrophenol reporter group measures two relevant pK_a values (Schemes 1 and 2 (18)). The lower pK_a is the pK_a for the ionization of the nitrophenol, and the higher pK_a is that for the dissociation of the adducted lysine residue. The measured pK_a values can be compared to pK_a values for model compounds in solution (17, 18). For example, the pK_a values for the reduced adduct of *N*-methylamine and 5-nitrosalicylaldehyde (*N*-methyl-2-hydroxy-5-nitrobenzylamine) are 5.9 and 10.7 (Scheme 2 (17)). Marked deviations from these pK_a values can be taken as evidence for perturbation effects of the protein on the chromophore. In this article, this approach is used to probe the nature of the active site of opsin and to place limits on the pK_a of the protonated form of the essential lysine.

EXPERIMENTAL PROCEDURES

Materials

Dodecyl β-D-maltoside (DM)[1] was obtained from Calbiochem. Pyridine/borane was a product of Aldrich, [^{14}C]formaldehyde was purchased from Du Pont-New England Nuclear, and Ammonyx LO was obtained from Onyx Chemical Co. Methyl α-D-mannopyranoside, concanavalin A-Sepharose 4B, Sephadex G-25, and dithiothreitol were obtained from Sigma. Bio-Gel HTP was a product of Bio-Rad. Hydrofluor (scintillation fluid) was obtained from National Diagnostics. The 5-nitrosalicylaldehyde was a product of Eastman Kodak Co., and the sodium borohydride was obtained from Fluka. 5-Nitrosalicyl alcohol was prepared by reducing the 5-nitrosalicylaldehyde with sodium borohydride. The dark-adapted retinas were purchased from the W. L. Lawson Co. Thin layer chromatography Silica Gel 60 F 254 sheets were products of E. Merck, West Germany. All other chemicals and solvents used were of the highest grade commercially available.

Methods

Preparation and Modification of Proteins—Rod outer segments and solubilized rhodopsin were prepared as already described by previously described procedures (19). Unless otherwise stated, rhodopsin modifications were carried out in a 10 mM PIPES, pH 6.5, buffer containing 100 mM NaCl and 6 mM DM (buffer A). All procedures involving the handling of nonbleached rhodopsin were conducted under dim red light. Rhodopsin and permethylated rhodopsin were stored at −70 °C between experiments.

Methylation Procedures—Rod outer segments were permethylated prior to rhodopsin isolation. This was accomplished by carrying out two rounds of methylation using 2 mM formaldehyde and 20 mM pyridine/borane. A stock solution of 0.5 M formaldehyde was prepared by hydrolyzing paraformaldehyde which was stored at 4 °C and used over a period of several months (20). Stock solutions of 2 M pyridine/borane were made up in 2-propanol and stored at −70 °C in aliquots of 1 ml. Rhodopsin concentrations were 40 μM on the basis of the absorbance at 500 nm using ϵ_{500} = 40,000 M^{-1} cm^{-1} (21). Each round of methylation of rhodopsin was allowed to proceed for 20–24 h at room temperature, whereupon the modified protein was isolated from the reaction mixture by diluting and centrifuging at 20,000 rpm for 30 min (J2-21, Beckman). The modified rhodopsin was then solubilized with dodecyl β-D-maltoside and chromatographed on a concanavalin A-Sepharose 4B column (1 ml gel for each 100 nmol of protein) equilibrated with buffer A containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ (buffer B), and the column was run at 10 ml/h taking 1-ml fractions. After washing with two column volumes, the protein was eluted with buffer B containing 0.5 M methyl α-D-mannopyranoside. The modified rhodopsin isolated was subjected to a third round of methylation using 500 μM formaldehyde and 10 mM pyridine/borane. This procedure produced a permethylated rhodopsin in which more than 95% of all available non-active-site lysines were dimethylated. The extent of lysine methylation was determined by further methylating with 2 mM [^{14}C]formaldehyde (1 Ci/mol) and 20 mM pyridine/borane for 48 h, with two additions of the reactants. Prior to scintillation counting, radiolabeled protein samples were dialyzed for at least 3 days against 10 mM HCl containing 0.5% Ammonyx LO (three changes of 500 ml for up to 1 ml of protein solution). This extra dialysis step was included to remove dissociable protein/formaldehyde products which are known to form readily (22).

Preparation of PMO—PMO was prepared by bleaching of permethylated rhodopsin under strong white light in the presence of 20 mM hydroxylamine. Bleached protein was isolated on a G-25 column equilibrated in buffer A taking 1-ml fractions and separated from the retinal oxime by chromatography on a hydroxylapatite column (10 nmol of protein/ml of gel) equilibrated in 20 mM potassium phosphate, pH 6.5, containing 100 mM NaCl and 6 mM DM. After making the sample 20 mM in potassium phosphate, the protein was loaded on the column, and the elution was accomplished using a gradient from 20 to 500 mM potassium phosphate (five times the column volume), pH 6.5, containing 100 mM NaCl and 6 mM DM.

Active-site-methylated PMO—The PMO was incubated in the presence of 20 mM pyridine/borane and 2 mM formaldehyde for 24 h at 4 °C, and several more additions of reactants were made until no regeneration of the protein was observed. The protein was separated from the reaction mixture by a Sephadex G-25 desalting column.

Modification of PMO with 5-Nitrosalicylaldehyde—The purified PMO was incubated with 10 mM 5-nitrosalicylaldehyde (NSA) at 4 °C until regeneration with 11-*cis*-retinal no longer occurred. An incubation period of 20 h was approximately what was required to achieve this level of modification. This incubation was followed by reduction with 10 mM sodium borohydride for 10 min at 4 °C. The protein concentrations used were routinely 3–5 μM based on the

[1] The abbreviations used are: DM, dodecyl β-D-maltoside; PMO, permethylated opsin; PMN, 5-nitrosalicylaldehyde modified permethylated opsin; PIPES, 1,4-piperazinediethanesulfonic acid; NSA, 5-nitrosalicylaldehyde.

absorbances at 280 and 500 nm (21). The protein was separated from the reaction mixture using a desalting column of Sephadex G-25, equilibrated in buffer A, and run taking 1-ml fractions. All the reactions and manipulations of the bleached protein were performed at 4 °C, because opsin was found to be much more susceptible than rhodopsin to thermal denaturation.

UV-visible Absorbance as a Function of pH—These experiments were performed by diluting 300 μl of NSA-opsin in 700 μl of 50 mM sodium phosphate buffer containing 6 mM DM at different pH values at 4 °C and recording the UV-visible spectra immediately between 650 and 250 nm. The spectra were recorded using a Perkin-Elmer UV-visible spectrophotometer thermostated at 4 °C. The pH values of the samples were measured on an Orion Research 601 A pH meter immediately before and after the spectra were recorded.

Stability of PMO as a Function of pH—The stability of PMO was measured as a function of pH. PMO samples were incubated at the different pHs used in the pH *versus* absorbance scans. The incubations were performed for the same periods of time required for the spectroscopic studies. Regeneration was allowed to occur with added 11-*cis*-retinal to determine the stability of the protein.

Regeneration of PMO—Modified PMO and controls were regenerated by adding a 5-fold molar excess of 11-*cis*-retinal in 2-propanol (volume of 2-propanol added being <1% v/v of the protein solution). Regeneration was complete after 30 min at 4 °C. Protein spectra (1 ml of solution) were recorded with a Perkin-Elmer λ 3B UV-visible spectrophotometer.

pK_a Determinations—The absorbance values were obtained from the spectroscopic data. An expression was written relating the observed optical density as a function of the pH in terms of the molar absorptivity for the basic and acid forms and the pK_a. Using the absorption values for each wavelength we obtain the best fit in each case, starting with approximate values of the molar absorptivity for the basic and acid forms and the pK_a. These approximate values correspond to the values obtained from the optical density at the extremes of pH used. An estimate of the pK_a can be read from the titration curve. An iterative nonlinear least squares regression method was used to approximate the parameters.

In much of the spectrum for which measurements are recorded, two ionizations may occur which require at least five unknown parameters. These consist of two pK_a values and the molar absorptivity for the three species. Good approximations to the extinction coefficients for the anionic and cationic forms can be made from the optical densities at high and at moderately low pH, and approximate values can be obtained from visual inspection of graphs of the data. An iterative computer program was used to produce the best values of the pK_a(s) at each wavelength, in order to find the best fit. For this process we used the commercially available program RS/1.

The equations used for the least squares treatment of the data were

$$A_T = \epsilon_a \frac{Z \cdot I \cdot J}{(X^2 + J)(X + I) \cdot J} + \epsilon_b \frac{X \cdot J \cdot Z}{(X^2 + J)(X + I) \cdot J} + \epsilon_c \frac{Z \cdot X^2}{(X^2 + J)(X + I) \cdot J} \quad (1)$$

and

$$A_T = \epsilon_a \cdot Z \frac{X}{K + X} + \epsilon_b \cdot Z \frac{K}{K + X} = Z \frac{(\epsilon_a X) + (\epsilon_b K)}{(K + X)} \quad (2)$$

where *a*, *b*, and *c* are the three species (anion, zwitterion, and cation), Z is the total protein concentration, X is the proton concentration, I, J, and K are the ionization constants, and ϵ_a, ϵ_b, ϵ_c are the extinction coefficients for the three species. A_T is the total absorption.

Equation 1 was used for the data fitting in the case of two pK_a values, and Equation 2 was used for the case of one pK_a.

RESULTS

Modification of PMO with 5-Nitrosalicylaldehyde—PMO was incubated with varying concentrations of 5-nitrosalicylaldehyde for 12 h and then reduced with $NaBH_4$ for 10 min. After chromatography, the abilities of these samples to regenerate with 11-*cis*-retinal were determined (Fig. 1*A*). As can be seen here, virtually complete inhibition of regeneration can be obtained at sufficiently high 5-nitrosalicylaldehyde concentrations. In a second set of experiments, the time course of modification was determined, using a fixed concentration of reagent (10 mM) for varying periods of time (Fig. 1*B*). A complete loss of regenerability was observed after 17–20 h at 4 °C. Higher borohydride concentrations and/or longer periods of reduction did not facilitate modification. These studies led to the adoption of a standard protocol for modification in which 5 μM PMO was incubated with 10 mM 5-nitrosalicylaldehyde for 17–20 h followed by incubation with 10 mM sodium borohydride for 10 min, all at 4 °C.

The next set of experiments was aimed at determining the stoichiometry and specificity of the modification reaction. A graph of the log of the half-life for regeneration inhibition as a function of the log of the 5-nitrosalicylaldehyde concentrations yields a straight line with a slope of approximately 1 (Fig. 2). This requires that the stoichiometry of adduct formation be one. Corroboration for this result comes from ^{14}C methylation studies. When PMO was methylated with [^{14}C] formaldehyde, 1.83 ± 0.17 methyl groups were incorporated, as expected. When the same methylation procedure was carried out on the modified PMO, only 0.67 ± 0.13 methyl groups were incorporated. Since the adduct is a secondary amine, up to one methyl group/opsin molecule could be incorporated. It should also be mentioned that the prior reduction of 5-nitrosalicylaldehyde by borohydride followed by incubation with PMO did not lead to any inhibition of regeneration of the latter with 11-*cis*-retinal.

Ultraviolet-visible Spectra of the Modified Opsin—The ultraviolet-visible spectrum of a sample of PMO modified as in Fig. 1*B* is shown in Fig. 3*A* at pH 6.5 and 9.8. At pH 6.5, the spectrum represents the protonated form of the adduct (17, 18). The spectrum of PMO incubated with NSA that had been prereduced with borohydride is identical to the ones shown in Fig. 3*B*. Clearly, there is little or no absorbance in the latter case. In Fig. 3*B* are shown spectra of PMO and PMO incubated with NSA but without including the borohydride reduction step. Again, no chromophore incorporation is observed. Finally, active-site dimethylated PMO treated as in Fig. 1*B* showed evidence for the incorporation of chromophore. These experiments, taken with those shown above, demonstrate the specificity of the modification procedure.

pK_a Measurements—These experiments depend on measuring the absorption spectra of PMN in the pH range where the protein is stable and not in a denatured form. This technique, then, can only supply limits to pK_a values when they are at the extremes of pH. In any case, it will supply important information on the active-site environment of the protein. In order to determine the stability range of the protein, PMO was incubated at varying pH values, and its ability to regenerate with added 11-*cis*-retinal was determined (Fig. 4). From this graph it is clear that the maximum range of stability of the protein is approximately from pH 5.5 to 10. The ultraviolet-visible spectra of PMN were then recorded over this pH range, and this data could be used to calculate the relevant pK_a values as described under "Methods" (17, 18). Unmodified PMO does not show any absorbance in the relevant regions of the spectrum, and hence the protein backbone does not interfere with the pK_a measurements. Approximate pK_a values could be determined by analysis of the data from the PMN spectra by determining each pK_a at the appropriate wavelength or determining both pK_a values at one wavelength. The fully protonated form of the reporter group absorbs maximally at 310 nm, the zwitterionic form absorbs maximally at 390 nm (ϵ = 18,100 M^{-1} cm^{-1}), and the anion absorbs maximally at 410 nm (ϵ = 20,000 M^{-1} cm^{-1}) (17). Initial experiments were performed to determine the spectra of PMN. As shown in Fig. 5, although a transition from the

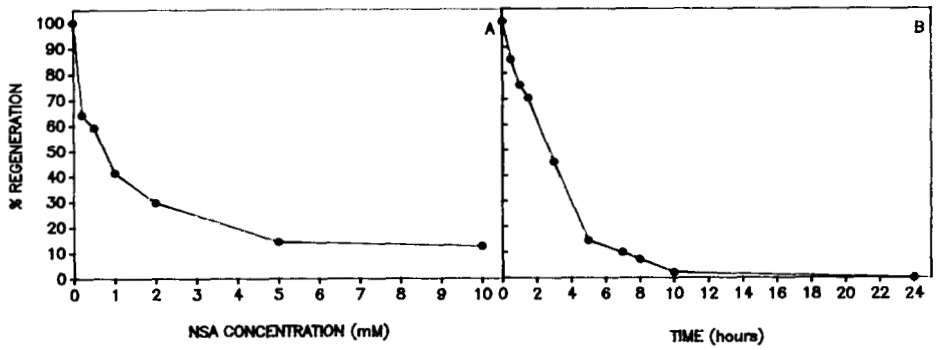


FIG. 1. **Inhibition of PMO regeneration by NSA.** *A*, PMO was incubated 12 h at 4 °C in the presence of varying NSA concentrations. *B*, samples of PMO were incubated in the presence of 10 mM NSA at different times. In both cases the incubation with NSA was followed by 10 min of reduction in the presence of sodium borohydride, and then PMO samples were separated from the reaction mixture on Sephadex G-25 desalting columns. The modified protein was incubated in the presence of 2-fold molar excess 11-*cis*-retinal to allow regeneration. The 100% regeneration was defined by the regeneration of appropriate controls.

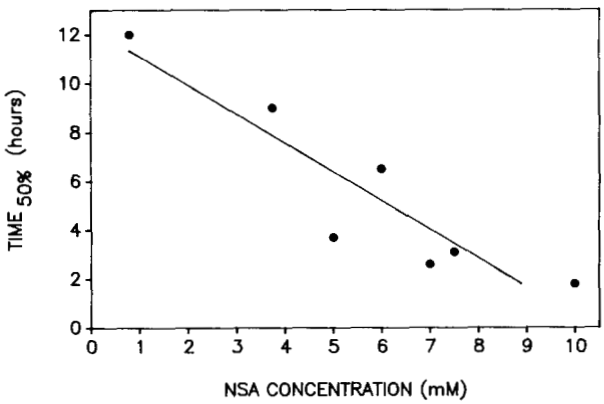


FIG. 2. **Kinetics of inhibition by NSA modification.** Samples of PMO were incubated in the presence of varying NSA concentrations at different times. The samples were then reduced for 10 min on ice in the presence of 10 mM sodium borohydride. The samples were separated from the reaction mixture on a Sephadex G-25 desalting column. The regeneration capacity of each sample was measured by incubation in the presence of 2-fold molar excess 11-*cis*-retinal for 1 h at 4 °C. A PMO control sample was regenerated under the same conditions. The half-time of regeneration loss was obtained from the time course curves at each concentration. The slope of the graph from plotting the NSA concentration *versus* the $t_{1/2}$ gives the number of NSA groups incorporated in the protein (n = 1.18).

310-nm state to the 390-nm state was observed, a 410-nm state was not formed even at extreme pH values. This means that the lysine dissociation was not observed and that its pK_a must be quite high and is certainly not abnormally low. To determine the pK_a(s) of the dissociation, changes in absorption spectra of the PMN were determined at 360, 390, and 430 nm at different pH values. The data thus obtained, along with estimates of the extinction coefficients of the absorbing species, were analyzed by an iterative computer program to fit the data to both one and two unique pK_a values. In this manner, precise pK_a values could be obtained rather than estimates. The data from this computer analysis are shown in Fig. 6 and fit a one pK_a model with high statistical probability ($p < 0.05$). The plotted curve shows one pK_a, and the data do not fit a two pK_a model with the same statistical probability as they fit the one pK_a model. Nevertheless two pK_a values can be calculated from these data (pK_{a1} = 7.68 and pK_{a2} = 8.49). However, in addition to being less statistically probable than the one pK_a model, a two pK_a model is inconsistent with the observed spectroscopic properties of the probe in which the NH ionization was not observed.

DISCUSSION

The studies reported here were aimed at gaining information about the active-site environment of opsin. It is thought that the Schiff base is found in one of the α-helical domains of rhodopsin which is buried in the membrane (1). One consequence of this is that a hydrophobic environment would be expected *per se* to have an important effect on the basicity of the buried lysine.

To obtain information on the active-site environment of opsin we made use of a reporter group technique, first applied by Westheimer (18) in a study of the pK_a of the active-site lysine of acetoacetate decarboxylase. In this technique, NSA is bound to the lysine in question via Schiff base formation and then fixed with sodium borohydride (17). Although this technique cannot be construed to be of general utility, it turns out to be applicable to the study of the active-site environment

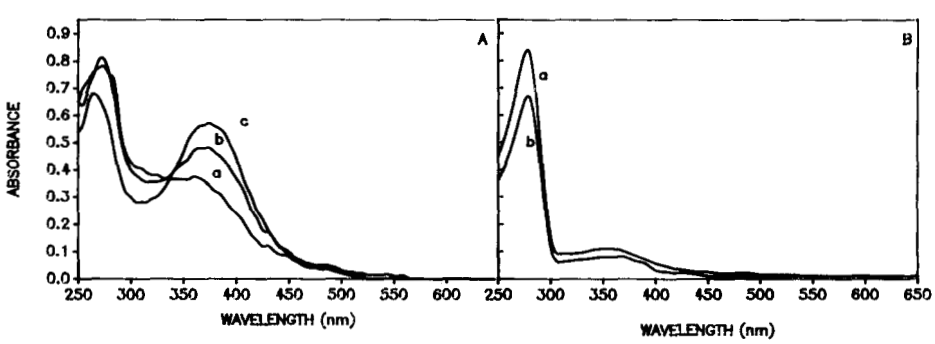


FIG. 3. **Spectra of different states of modification of PMO.** *A*, PMN samples at three pH values: *a*, pH 6.94; *b*, pH 7.81; *c*, pH 9.51. *B*, spectra of PMO: *a*, PMO spectrum at 4 °C, pH 6.5, before modification with NSA; *b*, active-site methylated PMO spectrum at 4 °C, pH 6.5, after incubation with NSA. The protein was separated from the reaction mixture on a Sephadex G-25 desalting column before the spectrum was taken.

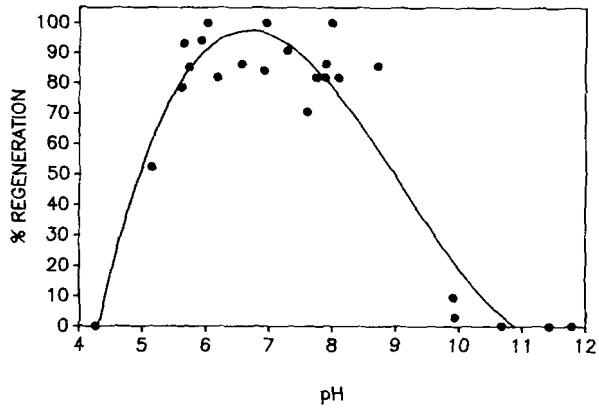


FIG. 4. **Study of the regeneration capacity of PMO at different pH values.** The PMO was regenerated for 1 h in the presence of 11-*cis*-retinal at 4 °C in buffers at different pH values. 100% regeneration was considered the regeneration of the control at pH 6.5.

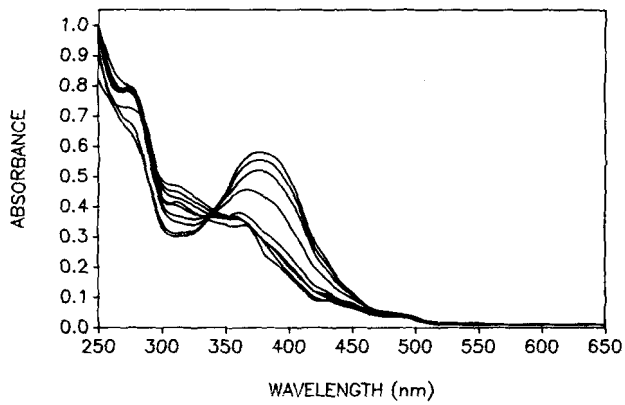


FIG. 5. **Variation of PMN with pH.** PMN was incubated at different pH values, and spectra of the samples were taken at 4 °C. The pH range used was from 5.5 to 9.5.

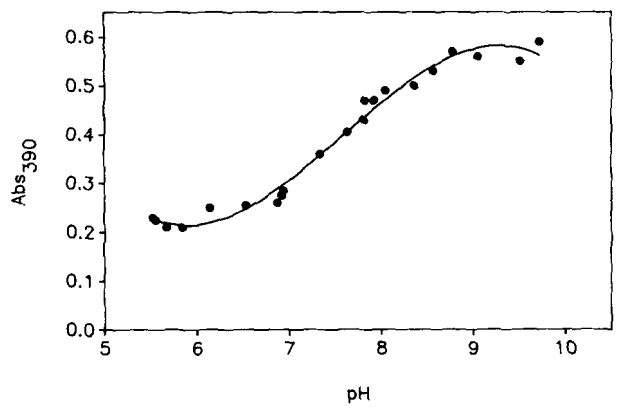


FIG. 6. **Titration curve of PMN at 390 nm.** The PMN was incubated at different pH values as described under "Experimental Procedures." Spectra at each pH were taken, and the absorbance values at 390 nm were obtained from those spectra.

of rhodopsin for two reasons. First, the 10 non-active-site lysine residues of rhodopsin can be reductively dimethylated to form permethylated rhodopsin, with little or no measurable alteration in the function of the protein (14). This treatment removes the non-active-site lysine residues from consideration when modifying the active-site lysine. Second, the resulting permethylated rhodopsin can be bleached to form PMO, which is susceptible to Schiff base formation at its active-site lysine with aromatic aldehydes (16). As demonstrated here, NSA was capable of forming a Schiff base with PMO, which could be reduced with sodium borohydride to form a stable adduct. The stoichiometry of the reaction could be determined to be one by the kinetics of the modification reaction (Fig. 3) and by the back titration of PMN with [^{14}C] formaldehyde. PMO incorporated approximately two methyl groups per opsin molecule, whereas PMN incorporated only approximately one. Furthermore, active-site dimethylated PMO did not incorporate reporter group at all. The conclusions from these studies were confirmed by spectroscopic studies on the adduct. Only PMN itself showed the expected absorption profile. Neither PMO by itself nor dimethylated PMO (or permethylated rhodopsin), both further processed with NSA followed by reduction, showed any absorption above 300 nm. Furthermore, prior reduction of NSA with borohydride followed by the addition of PMO did not lead to the incorporation of reporter group into the latter after chromatography. These experiments, in total, allow for the conclusion that a single reporter group has been incorporated at the active-site lysine of PMO.

Having demonstrated that the PMN contained the reporter group properly adducted to the active-site lysine, it was possible to begin the measurements on the pK_a values of the chromophore. Absorption spectra of PMN at pH values ranging from 5 to 10 showed a single spectroscopic shift from 310 to 385 nm. A further shift to approximately 410 nm was not observed at the accessible pH values. This means that the NH dissociation does not occur under the conditions of the measurements. The data indicate that only the phenolic dissociation occurs. To accurately determine this pK_a, measurements had to be made at several wavelengths (360, 390, and 430 nm), and the resulting data have to be analyzed by computer (17, 18). Equations were derived which relate the observed optical densities, the extinction coefficients of the acid and base forms of the "indicator," and the pK_a values at each individual pH (18). An iterative commercially available program was then used to accurately determine the pK_a values in question. The data were fit to both a two pK_a and a one pK_a model. The fit to a model in which there is one pK_a = 7.84 ± 0.19 was much better than the fit to a model in which there were two pK_a values. This, of course, is consistent with the spectroscopic studies in which the 410-nm absorbing species was not observed. The pK_a measured is ascribed to the phenol group because its value is very similar to that approximated by measuring the fall of the absorption of PMN at 310 nm as a function of pH. Only the acid form of the chromophore absorbs at this wavelength (17). The measured pK_a of the phenol is actually increased by approximately 2 units over the model compound, which has a lower pK_a of approximately 5.9 (17). That is, the phenol has been rendered less acidic by virtue of its being adducted to the active-site lysine of opsin. At the same time, however, the amino group is being rendered apparently less acidic than the model compound.

The shifts in pK_a could be caused by either charge or hydrophobic effects. Simple electrostatic considerations show that either a positive or a negative charge at the active site would have the effect of moving both pK_a values in the same direction (18). A negatively charged amino acid at the active site would decrease the acidities of both the phenol and the protonated lysine moieties. A positively charged amino acid at the active site would increase the acidities of the phenol and the protonated lysine moieties together. On the other hand, a hydrophobic environment would decrease the acidity of the phenol moiety but increase it for the protonated lysine

moiety. On the basis of this discussion, a model in which the active-site lysine is adjacent to a negatively charged amino acid would best fit the available data. Of course, this negative charge could be the same one as the counterion to the protonated Schiff base of rhodopsin.

It is clear that the pK_a of the protonated Schiff base of rhodopsin must be quite high. This is known because protonation of this site is key to the absorption spectrum of rhodopsin (11). Unprotonated Schiff bases absorb light in the 350-nm range (23). Rhodopsin will denature at high pH values before its absorption spectra can be shifted to the blue. The work reported here also cannot measure a pK_a value for the active-site lysine of the modified opsin, but it is certainly not unusually low, as found with certain other catalytically active lysine residues. For example, the pK_a of the active-site lysine of acetoacetate decarboxylase is approximately 6 (18). This low pK_a is presumably selected for because the function of the active-site lysine in this enzyme is to engage in facile Schiff base formation with its substrate acetoacetate (18). Assuming that the pK_a of lysine of opsin is similar to that of PMN, then it is difficult to understand how ready Schiff base formation occurs between opsin and 11-*cis*-retinal. This is because, at pH 6.5, a moiety with a p$K_a > 10.5$ would have only one part in 10,000 in the neutral form capable of engaging in Schiff base formation. Of course, it is quite possible that a conformational transition driven by the binding of 11-*cis*-retinal substantially lowers the pK_a of the active-site lysine. However, if this were true, then one would have to explain why the Schiff base itself is so basic.

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